Biochemical and Biophysical Research Communications 443 (2014) 62–67

Contents lists available at ScienceDirect

Biochemical and Biophysical Research Communications

journal homepage: www.elsevier.com/locate/ybbrc

βTrCP-mediated ubiquitylation regulates protein stability of Mis18β in a cell cycle-dependent manner

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ARTICLE INFO

Article history:
Received 7 November 2013
Available online 21 November 2013

Keywords:
Mis18β
βTrCP
Ubiquitylation
Protein stability
Mitosis
Centromere

ABSTRACT

Ubiquitin E3 ligases including SCF complex are key regulators of cell cycle. Here, we show that Mis18β, a component of Mis18 complex governing CENP-A localization, is a new substrate of βTrCP-containing SCF complex. βTrCP interacted with Mis18β exclusively during interphase but not during mitosis and mediated proteasomal degradation of Mis18β leading to the inactivation of Mis18 complex during interphase. In addition, uncontrolled stabilization of Mis18β caused cell death. Together, we propose that βTrCP-mediated regulation of Mis18β stability is a mechanism to restrict centromere function of Mis18 complex from late mitosis to early G1 phase.

1. Introduction

Cell cycle is tightly regulated by various protein complexes which repress or activate specific proteins such as CDKs (cyclin-dependent kinases) [1]. If this regulation fails, the cells are either arrested to specific phase of cell cycle or undergo uncontrolled proliferation which might cause cancer [2,3]. Cell cycle progression is mostly achieved by changing the stability of regulatory proteins through ubiquitin-dependent proteasomal degradation [4–6]. Deregulation of protein stability responsible for the transition of cell cycle phase leads to cell cycle arrest and apoptotic cell death [7,8]. In line with this regulation, it is obvious that many proteins for phase transition in cell cycle turned out to be tumor suppressors [9].

Cullin-based E3 ligases belong to the RING-H2 E3 ligase subfamily and recognize various substrates involved in cell cycle regulation by changing a variable, substrate-recognizing component in the ligase complex [10]. Among Cullin-based complexes, SCF complexes (Skp1/Cul1/F-box protein) are well-characterized, and F-box proteins determine its substrate specificity [11]. As an F-box protein, Skp2 targets CDK inhibitors such as p27 and p21, and promotes CDK activity during G1/S transition [12]. Another F-box protein, βTrCP plays a role in controlling G2/M transition by recognizing a CDK1 inhibitor, WEE1, and in regulating APC/C activity through an APC inhibitor, EMI1 [7]. As these F-box proteins usually target tumor suppressors, verification of substrates is important for cancer study.

The regulation of chromosome segregation during mitosis is another layer of cell cycle regulation. There are fine tuning mechanisms governing centromere function and CENP-A localization [13,14]. Recently, we reported that Mis18α, a component of the Mis18 complex (Mis18α/Mis18β/Mis18BP1), participates in maintaining DNA methylation pattern on centromere region at the specific stage of cell cycle, from late mitosis to early G1, by recruiting DNMT3A/3B, which is critical for centromeric localization of newly synthesized CENP-A [15]. Although we elucidated underlying functional mechanism of Mis18 complex in centromere, it is not clear how the function of Mis18 complex in centromere is restricted to specific cell cycle phases, particularly from late mitosis to early G1 [16]. Recent report showed that massive phosphorylation of Mis18BP1 by CDK1/CDK2 during interphase blocked centromere localization of Mis18 complex, suggesting that it might be one mechanism to keep Mis18 complex out of centromere [17].

In this study, we figured out the functional mechanisms of mitosis-specific regulation of Mis18β. We found that the protein stability of Mis18β was increased in mitosis but, significantly diminished during interphase. Further, we also demonstrated that $SCF^{\beta TrCP}$ complex functioned as an ubiquitin E3 ligase mediating proteasomal degradation of Mis18β during interphase. Finally, we elucidated that the regulation of protein level of Mis18β was crucial for proper cell growth.

Abbreviations: SCF, Skp1/Cul1/F-box protein complex; CDK, cyclin-dependent kinase; CHX, cycloheximide; H3S10ph, phosphorylation at 10th serine residue of histone H3; aa, amino acid.

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http://dx.doi.org/10.1016/j.bbrc.2013.11.058

2. Materials and methods

2.1. Plasmid construction

Deletion mutants of Mis18α, D1 (38–204 aa) and D2 (119–204 aa) were described in previous report [15]. Mis18α-CA mutants (Mis18α-CA1, Mis18α-CA2, and Mis18α-CA3) were generated by site-directed mutagenesis using nPfu-Forte DNA polymerase (Enzynomics, South Korea) and CA1, 3 mutant was generated by repeating mutagenesis step for CA3 using CA1 as a template. All mutants were verified by sequencing.

2.2. Immunocytochemistry and TUNEL assay

The cells grown on coverslips were washed three times with PBS and then fixed with 2% paraformaldehyde in PBS for 10 min at room temperature. Fixed cells were permeabilized with 0.5% Triton X-100 in PBS (PBS-T) for 10 min at room temperature. Blocking was performed with 10% bovine serum in PBS-T for 30 min. For staining, cells were incubated with anti-Flag M2 IgG (Sigma–Aldrich, USA) overnight at 4 °C, followed by incubation with fluorescent labeled secondary antibodies for 1 h at room temperature (Invitrogen, USA). The TUNEL assay was performed according to the manufacturer's instructions (Roche, Germany).

2.3. Ubiquitylation assay

Ubiquitylation assay was performed as described previously [18]. Briefly, cells were transfected with a combination of plasmids including plasmid expressing HisMax-ubiquitin. After incubation, cells were treated with 10 μM MG132 for 6 h, lysed and incubated with Ni^{2+}-NTA beads (QIAGEN, Germany). After washing the beads, bound proteins were eluted by 200 mM imidazole and subject to immunoblot analysis.

2.4. RNA interference by siRNA and shRNA

For knock-down of specific genes, cells were transfected with siRNA or shRNA as indicated in figures and harvested for experiments 72 h after transfection. The target sequence of siRNA or shRNA against endogenous βTrCP or Mis18α are as follows: shMis18α (human), 5′-GGAACAGAAGCUAUCCAAA-3′; siβTrCP (human), 5′-GUGGAAUUUGUGGAACAUC-3′ [19].

2.5. Cell cycle synchronization

HeLa cells stably expressing either Flag-Mis18α or Flag-Mis18β were synchronized at G1/S phase and released as described previously [15]. For G2/M arrest, cells were incubated with media containing 0.4 μg/ml nocodazole for 12 h and harvested for experiment.

2.6. Statistical analysis

Statistical differences in test and control samples were determined by Student's *t*-test using the Statview package (Abacus Concepts, Inc., USA).

3. Results

3.1. The protein stability of Mis18β is regulated in a cell cycle-dependent manner by ubiquitin–proteasome system

Although we reported that centromeric deposition of CENP-A was regulated by Mis18/DNMT3A/3B complex during mitosis [15], the reason why Mis18 complex could regulate centromere function only during mitosis is still questionable. Given that Mis18 complex does not function during interphase, we investigated the protein levels of Mis18α and Mis18β during cell cycle. Cells were synchronized using double thymidine block, collected in specific stages of cell cycle depending on release time, and then protein levels of Mis18α and Mis18β were measured from cell extracts. Interestingly, we found that Mis18β level was increased during mitosis, overlapping with phosphorylation of H3S10 (Fig. 1A), while Mis18α protein level remained unchanged during cell cycle (Fig. S1A). We detected increased protein level of Mis18β again when the cells were synchronized in mitosis by treating with nocodazole compared to asynchronous cells (Fig. 1B). Based on these results, we hypothesized that the fluctuation of Mis18β protein level during cell cycle might be due to the regulation of Mis18β stability. To prove this, we first examined the stability of Mis18β by treating cells with cycloheximide (CHX), an inhibitor of protein synthesis, and examined protein turnover rates. Mis18β degraded rapidly for short time after CHX treatment (within 2 h) (Fig. 1C). The stability of Mis18β was significantly increased after blocking proteasome activity by MG132 treatment (Fig. 1D), suggesting that proteasomal degradation is responsible for regulation of Mis18β stability. In addition, we found that ubiquitylation of Mis18β was significantly increased in the cells without mitotic arrest, whereas the ubiquitylation of Mis18β was decreased in mitotic cells (Fig. 1E). Together, these findings suggest that the protein level of Mis18β remained low during interphase through ubiquitylation-dependent proteasomal degradation.

3.2. $SCF^{\beta TrCP}$ is an E3 ligase complex targeting Mis18β

Next, we screened for candidate ubiquitin ligases which are responsible for the regulation of Mis18β stability. Since

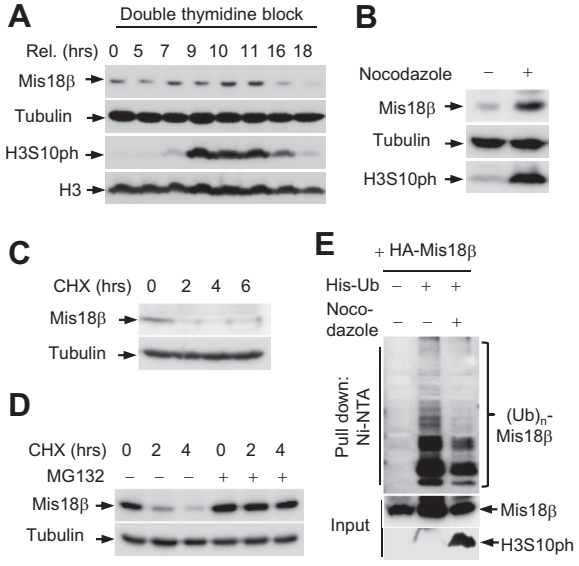

Fig. 1. Cell cycle-dependent, ubiquitin-mediated degradation of Mis18β. (A) HeLa cells stably expressing Flag-Mis18β were synchronized by double thymidine block, released, and harvested at indicated time points. Mitosis was determined by the level of H3S10ph. (B) The protein level of Mis18β was measured from asynchronous or nocodazole-arrested mitotic HeLa cells stably expressing Flag-Mis18β. (C) Cycloheximide (CHX) treated HeLa cells were collected at indicated time points and protein level of Flag-Mis18β was measured. (D) Mis18β stability was measured as in (C) in the absence or presence of proteasome inhibitor, MG132. (E) HeLa cells were transfected with plasmids expressing HA-Mis18β and HisMax-Ubiquitin. After 36 h of transfection, cells were treated with MG132 for 6 h. Ubiquitylated proteins were pulled down with Ni^{2+}-NTA beads and Mis18β was visualized by immunoblotting with anti-Flag antibody. Mitotic arrest was obtained by nocodazole treatment and confirmed by detecting H3S10ph.

Cullin-based E3 ligases are known to play critical roles in governing cell cycle progression, we first tested the interaction between Mis18β and a series of Cullin-based E3 ligases [6]. Co-immunoprecipitation assays revealed Cul1, Cul3 and Cul4B as putative interacting partners of Mis18β among 6 Cullin proteins tested: Cul1, Cul2, Cul3, Cul4A, Cul4B, and Cul5 (Fig. S1B). We then examined whether the ectopic expression of each Cullin protein and its F-box partner can increase ubiquitylation of Mis18β. Interestingly, ubiquitylation of Mis18β was significantly increased when Cul1 was introduced with βTrCP but not with another F-box protein, Skp2 (Fig. 2A). On the contrary, expression of neither Cul3/SPOP nor Cul4B/DDB1 increased ubiquitylation of Mis18β. The specificity of βTrCP to Mis18β was confirmed further by checking its interaction with Mis18β. In consistent with ubiquitylation assay, βTrCP, but not Skp2, interacted with Mis18β (Fig. 2B). In addition, the interaction between Mis18β and βTrCP was reduced in cells arrested in mitosis compared to asynchronous cells (Fig. 2C), indicating that the stabilization of Mis18β in mitosis is due to the reduced interaction between βTrCP and Mis18β.

To further verify the critical role of βTrCP in the regulation of Mis18β stability, we checked both ubiquitylation and the protein level of Mis18β after knock-down of βTrCP. The ubiquitylation of Mis18β was significantly reduced by siRNA against βTrCP (Fig. 2D). Further, knock-down of βTrCP stabilized Mis18β in cells that are stably expressing Flag-Mis18β (Fig. 2E). Since mitosis specific H3S10ph was not detected in βTrCP knock-down cells, we could eliminate the possibility that stabilization of Mis18β is due to unexpected mitotic arrest in βTrCP knock-down cells (Fig. 2E). We, therefore, concluded that the proteasomal degradation of Mis18β was mediated by the Cul1-based ubiquitin E3 ligase complex containing βTrCP as a Mis18β-specific F-box protein.

3.3. CxxC motif of Mis18α is critical region for stabilizing Mis18β

Mis18β could be in a complex with Mis18α and Mis18BP1 during late mitosis to early G1, since all of these three proteins were found in the same location of centromere as dots at these stages of cell cycle and knock-down of any Mis18 protein affected centromere localization of other proteins [16,20]. To examine whether the mutual interaction of Mis18 proteins influences Mis18β stability, we transfected cells with fixed amount of plasmids expressing Mis18β and controlled the expression of Mis18α in the same cells either by increasing the amount of Mis18α plasmid or by introducing shRNA targeting Mis18α, and measured the expression level of Mis18β. Interestingly, the protein level of Mis18β was well-overlapped with the expression level of Mis18α (Fig. 3A). Further, ubiquitylation of Mis18β was diminished by Mis18α, whereas knock-down of Mis18α greatly increased ubiquitylation of Mis18β (Fig. 3B). These data indicate that Mis18β was stabilized in the presence of Mis18α.

Next, to elucidate which region of Mis18α is determinant for Mis18β stability, we tested turnover rate of Mis18β in the presence of various deletion mutants of Mis18α. The results indicated that the middle region Mis18α harboring cysteine rich region is important for the stabilization of Mis18β (Fig. S2). Since two CxxC domains in cysteine rich region of Mis18α has shown to be important for the function of Mis18 complex [16], we hypothesized that the CxxC domains of Mis18α are critical for Mis18β stability. To verify this possibility, we generated three CxxC domain mutants of Mis18α (i.e. CA1, CA2, and CA3), as indicated in Fig. 3C by replacing two cysteine residues to alanines, and tested their contribution to Mis18β stability. Interestingly, two CA mutant forms of Mis18α, CA1 and CA3, which were known to be defective in supporting centromere localization of CENP-A [16], were not able to stabilize Mis18β, while CA2 form of Mis18α, which is still functional for CENP-A loading, increased Mis18β stability (Fig. 3C). Furthermore, Mis18α CA1 and CA3 forms did not protect Mis18β from ubiquitylation compared to wild-type and CA2 forms of Mis18α (Fig. 3D). Taken together, our results indicate that the function of Mis18 complex supporting centromeric localization of CENP-A and the regulation of Mis18β stability by Mis18α is required based on the overlapping effects of CA mutant forms of Mis18α on both cases.

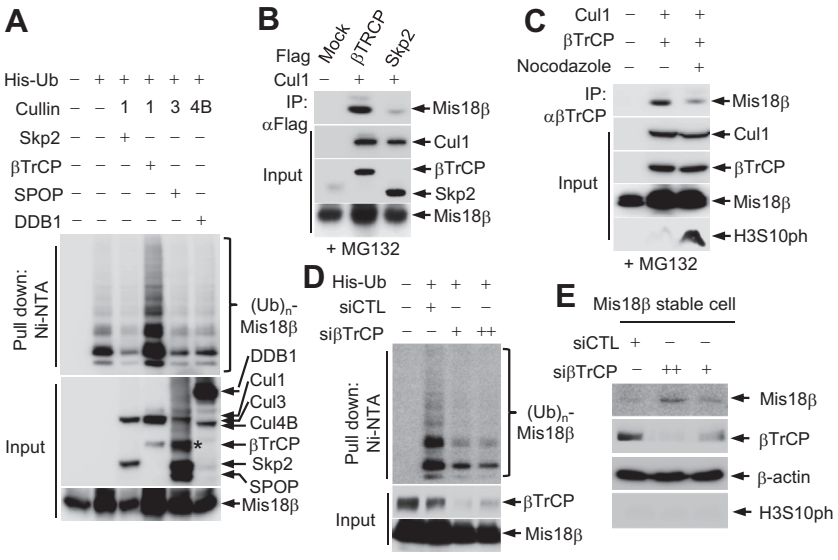

Fig. 2. Cul1/βTrCP recognized and degraded Mis18β. (A) Ubiquitylation assay of Mis18β with several E3 ligase complexes. Plasmids expressing E3 ligase complexes (Cul1/Skp2, Cul1/βTrCP, Cul3/SPOP, and Cul4B/DDB1) were transfected with plasmid expressing HA-Mis18β in HeLa cells. Ubiquitylation of Mis18β was measured as described in Fig. 1E. (B) Plasmids expressing Flag-βTrCP/Cul1 or Flag-Skp2/Cul1 were transfected with plasmid expressing HA-Mis18β in HeLa cells. After immunoprecipitation (IP) with anti-Flag antibody, Mis18β was visualized by immunoblotting. (C) IP was performed with the cell extracts generated from asynchronous or mitotically arrested cells with nocodazole. Mitotic arrest was confirmed by immunoblot of H3S10ph. (D) Ubiquitylation of Mis18β was measured as described in Fig. 1E from the cells transfected with control or βTrCP-specific siRNA for 48 h. (E) The protein level of Mis18β was determined by immunoblotting in cells stably expressing Flag-Mis18β transfected with βTrCP-specific siRNA.

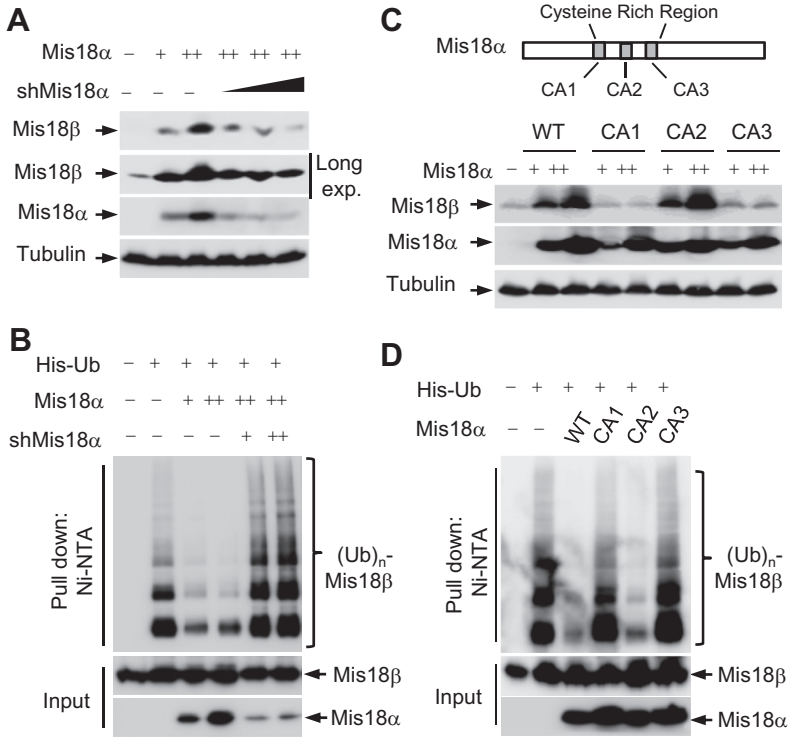

Fig. 3. Mis18α stabilized Mis18β through its CxxC motifs. (A) Fixed amount of plasmid expressing Mis18β was transfected in HeLa cells with increasing amount of Mis18α-expressing plasmid. This increased Mis18α level was reduced to basal level by co-transfecting with Mis18α-specific shRNA. (B) Ubiquitylation of Mis18β was measured as described in Fig. 1E in the presence of varying amount of Mis18α. (C) Upper schematics depicted three CxxC motifs (shaded box) within Mis18α. Cysteine to alanine mutation of each CxxC motif was sequentially represented as CA1, CA2, and CA3. The stabilization of Mis18β was measured after transfecting plasmid expressing Mis18β with wild-type or each mutant form of Mis18α as shown in the bottom. (D) Ubiquitylation of Mis18β was measured in the presence of wild-type or each mutant form of Mis18α.

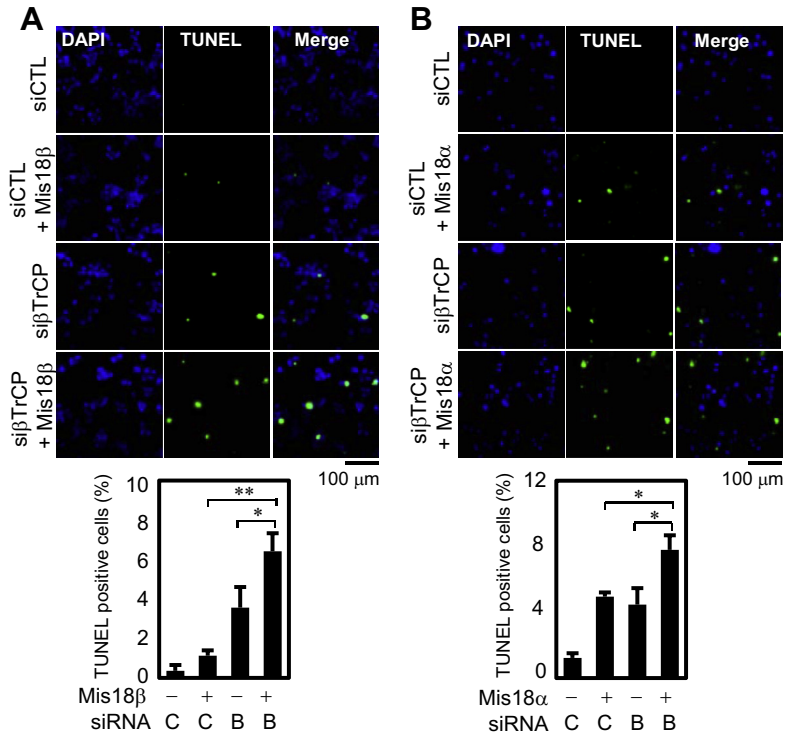

Fig. 4. Deregulation of Mis18β stability during cell cycle led to apoptotic cell death. (A) TUNEL assay was performed using the cells transfected by Mis18β-expressing plasmid with control siRNA (C) or with siRNA for βTrCP (B). After 48 h of transfection, apoptotic cells were visualized by TUNEL staining. Bottom histogram represented percentages of TUNEL positive cells. (B) TUNEL assay was performed as in (A) but in the presence of Mis18α instead of Mis18β.

3.4. Regulation of Mis18β stability during cell cycle is critical for proper centromere function

Because any component of Mis18 complex is indispensable for CENP-A localization to centromere, we hypothesized that the maintenance of Mis18β at low level during cell cycle of other than mitosis might be important to ensure proper cell division by prohibiting non-specific deposition of CENP-A. Since stabilization of CENP-A by deleting its ubiquitin E3 ligase, psh1, mediated cell death through altered CENP-A accumulation in euchromatin [21], we measured cell viability using TUNEL assay in a condition that Mis18β is stabilized in interphase cells by knocking-down βTrCP. As shown in Fig. 4A, the number of TUNEL-positive cells increased by an average of 4-fold after knock-down of βTrCP. In addition, ectopic expression of Mis18β along with knock-down of βTrCP further increased cell death by an average of 6-fold. Ectopic expression of Mis18β without knock-down of βTrCP showed little effect on cell death (Fig. 4A and Fig. S3A). To further confirm the importance of Mis18β regulation in cell division, we stabilized Mis18β by overexpressing Mis18α. The number of TUNEL-positive cells increased by an average of 5-fold as Mis18α was overexpressed and cell death was even more severe (8-fold increase compared to control) when Mis18α overexpressing cells were additionally transfected with siRNA against βTrCP (Fig. 4B and Fig. S3B). Taken together, these results indicate that uncontrolled stabilization of Mis18β affects proper progression of cell cycle probably by causing mis-localization of CENP-A leading to mis-segregation of mitotic chromosomes.

4. Discussion

Proper formation and maintenance of centromere is a critical part of genome stability. One way of maintaining centromere integrity is keeping CENP-A level tightly regulated by ubiquitin-mediated proteolysis to prohibit overspreading or off-site formation of centromere. It has been shown that CENP-A is overexpressed in many cancer tissues and cell lines and knock-down of CENP-A to normal level significantly reduced number of aneuploid cells [22,23]. Recent studies identified a CENP-A-specific ubiquitin E3 ligase Psh1 in yeast and a CENP-A-recognizing F-box protein Ppa for SCF complex in Drosophila [21,24,25]. In addition to directly controling CENP-A level, centromere integrity seems to be achieved by regulating proteins involved in CENP-A deposition. Similar to CENP-A itself, a CENP-A-specific histone chaperone HJURP was found to be overexpressed in many types of cancers, and overexpression of HJURP caused mitotic defects in human cell lines [26,27]. In addition, cell cycle-dependent, CDK1/CDK2-mediated phosphorylation of Mis18BP1 kept Mis18 complex out of centromere to ensure the proper timing of CENP-A loading during cell cycle [17].

In this report, we showed that the protein stability of Mis18β is actively regulated by $SCF^{\beta TrCP}$ complex during interphase. Given that the centromeric localization of Mis18 complex was found only from late mitosis to early G1 phase of cell cycle and all three components were required for the centromeric localization and the function of Mis18 complex [16], we suggest that keeping Mis18β level low is an additional way of maintaining centromere integrity. With a low level of Mis18β, Mis18 complex is probably not fully functional during interphase. On the other hand, overexpression of Mis18α/Mis18β or stabilization of Mis18β by knock-down of βTrCP induced cell death, which is similar to the case of CENP-A overexpression. We also demonstrated that the stability of Mis18β is increased in the presence of Mis18α and that the CxxC motifs in Mis18α are critical for the stabilization of Mis18β. Since the CxxC mutant forms of Mis18α were unable to localize on centromere [16], we speculate that the stabilization of Mis18β is an important process for centromeric localization of Mis18 complex.

$SCF^{\beta TrCP}$ is well known regulator for CDK1-dependent cell cycle entry and exit by activating APC/C function. Although βTrCP has a major role in mitotic entry, it also has a broad range of substrates regulating cell survival and growth. Specific targeting of $SCF^{\beta TrCP}$ to Mis18β during interphase would be a way to secure cell cycle by inhibiting unscheduled CENP-A localization. βTrCP interacted with Mis18β preferentially in interphase but much weaker in mitosis in our study. Since phosphorylation mediates recognition by βTrCP, identification of phosphorylation site(s) on Mis18β and responsible kinase will be one of the important future studies to establish more detailed mechanisms.

Conflict of interest

The authors declare no conflict of interest.

Acknowledgments

We thank K.E. Pyo for critical reading of our paper. This research was supported by Basic Science Research Program (2011-0006660) through NRF Grant funded by the Korea government (MEST) to K.I.K., by the NRF Grant funded by the Korea government (MSIP) (2011-0030074) to R.J., and by the Korean Health Technology R&D Project, Ministry of Health, Welfare & Family Affairs (A121854) to I.S.K.

Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.bbrc.2013.11.058.

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